JC20 Rec'd PCT/PTO 19 FEB 2002

FORM PTO-1390 U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE (MODIFIED)	ATTORNEY'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371	X-13268
	U S. APPLICATION NO. (if known, see 37 C.F.R. 1.5) 10/069385

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US00/20806	**08/31/2000 (08.31.00)**	**09/10/1999 (09.10.99)**

TITLE OF INVENTION: FLINT ANALOG COMPOUNDS AND FORMULATION THEREOF

APPLICANT(S) FOR DO/EO/US: Paul Robert Atkinson, et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] has been transmitted by the International Bureau.
   c. [X] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [X] A copy of the International Preliminary Examination Report (IPER), including any annexes, and, if not in English, an English language translation of the annexes to the IPER under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [X] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [ ] Other items or information:

U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
10/069385	PCT/US00/20806	X-13268

17. [X] The following fees are submitted:

CALCULATIONS PTO USE ONLY

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO. **$1040.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO..... **$750.00**

International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**

International preliminary examination fee paid to USPTO (37 CRF 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) **$100.00**

ENTER APPROPRIATE BASIC FEE AMOUNT =				$ 890.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than __20 __30 months from the earliest claimed priority date (37 CFR 1.492(e)).				$	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	8 - 20=		**X $18.00**	$	
Independent claims	1 - 3=		**X $84.00**	$	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			**+ $280.00**	$	
TOTAL OF ABOVE CALCULATIONS =				$ 890.00	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).				$	
SUBTOTAL =				$ 890.00	
Processing fee of **$130.00** for furnishing English translation later than __ 20 __30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
TOTAL NATIONAL FEE =				$	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$	
TOTAL FEES ENCLOSED =				$ 890.00	
				Amount to be refunded	$
				charged	$

a. [ ] A check in the amount of $_____ to cover the above fees is enclosed.
b. [X] Please charge my Deposit Account No. 05-0840 in the amount of $ 890.00 to cover the above fees. A duplicate copy of this sheet is enclosed.
c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 05-0840. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
ELI LILLY AND COMPANY
PATENT DIVISION/XXX
LILLY CORPORATE CENTER

_____
Date

39,872
REGISTRATION NUMBER

Thomas D Webster
SIGNATURE

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PATENT TRADEMARK OFFICE

"Express Mail" mailing label number EL832892408US

Date of Deposit Feb. 19, 2002

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Arlington, VA 22202.

Queen Thomas
Printed Name

Queen Thomas
Signature

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

The Accompanying Application

Applicants : Paul Robert Atkinson, et al.

For : Flint Analog Compounds and Formulation Thereof

Docket No. : X-13268

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents

Arlington, VA 22202

Sir:

Please amend the accompanying application as follows:

In the Claims

Please delete claims 1-13 and add the following 14-21:

14. A composition comprising a divalent metal cation and a protease-resistant FLINT analog.

15. A composition as in claim 14, wherein said cation is selected from the group consisting of Zn^{+2} , Ca^{+2} , Ni^{+2} Mn^{+2}, Fe^{+2}, Co^{+2}, and Cd^{+2}.

16. A composition of Claim 15, wherein the divalent metal cation is Zn^{+2}.

17. A composition of Claim 14, wherein the FLINT analog is resistant to proteolysis at position 218 of SEQ ID NO:1.

18. A composition of Claim 17, wherein the arginine at position 218 of SEQ ID NO:1 is substituted by glutamine.

19. A pharmaceutical formulation comprising a composition of Claim 18 in combination with one or more pharmaceutically acceptable carriers, diluents, or excipients.

20. A formulation of Claim 19, wherein the total cation concentration is about 0.001 to 5.0 mg/mL.

21. A formulation of Claim 20, wherein the total cation concentration is about 0.05 to 1.5 mg/mL.

Respectfully submitted,

Howard Webster

Thomas D. Webster
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Phone: 317-276-3334

Eli Lilly and Company
Patent Division/TDW
Lilly Corporate Center
Indianapolis, Indiana 46285

February 19, 2002

What is claimed is:

14. A composition comprising a divalent metal cation and a protease-resistant FLINT analog.

15. A composition as in claim 14 wherein said cation is selected from the group consisting of Zn^{+2} , Ca^{+2} , Ni^{+2} Mn^{+2}, Fe^{+2}, Co^{+2}, and Cd^{+2}.

16. A composition of Claim 15, wherein the divalent metal cation is Zn^{+2}.

17. A composition of Claim 14, wherein the FLINT analog is resistant to proteolysis at position 218 of SEQ ID NO:1.

18. A composition of Claim 17 wherein the arginine at position 218 of SEQ ID NO:1 is substituted by glutamine.

19. A pharmaceutical formulation comprising a composition of Claim 18 in combination with one or more pharmaceutically acceptable carriers, diluents, or excipients.

20. A formulation of Claim 19, wherein the total cation concentration is about 0.001 to 5.0 mg/mL.

21. A formulation of Claim 20, wherein the total cation concentration is about 0.05 to 1.5 mg/mL.

FLINT ANALOG COMPOUNDS AND FORMULATIONS THEREOF

Background of the Invention

The present invention is in the field of human medicine, particularly in the treatment and prevention of disorders that may be associated with the binding of FasL to the Fas receptor. More specifically, the present invention relates to compounds and formulations of a FLINT analog.

A number of tumor necrosis factor receptor proteins ("TNFR proteins") and proteins homologous thereto have been isolated in recent years. They have many potent biological effects and aberrant activity of these proteins has been implicated in a number of disease states.

One such TNFR homologue, reported in July, 1998 (Gentz *et al.*, WO 98/30694), binds the protein FAS Ligand and thereby inhibits the activation of another TNFR homologue, FAS, by FAS Ligand (U.S. Provisional Applications Serial Nos. 60/112,577, 60/112,933, and 60/113,407, filed December 17, 18 and 22, 1998, respectively). This new protein is referred to herein as "FAS Ligand Inhibitory Protein" or "FLINT."

Over activation of FAS by FAS Ligand has been implicated in a number of pathological conditions, including runawayapoptosis (Kondo *et al.*, Nature Medicine 3(4):409-413 (1997)and Galle *et al.*, *J. Exp. Med.*182:1223-1230 (1995)) and inflammatory disease resulting from neutrophil activation (Miwa *et al.*,. *Nature Medicine 4:*1287 (1998)).

"Runaway apoptosis" is a level of apoptosis greater than normal or apoptosis occurring at an inappropriate time. Pathological conditions caused by runaway apoptosis include organ failure, for example in the liver, kidneys and pancreas. Inflammatory diseases

associated with excessive neutrophil activation include, but are not limited to, sepsis, ARDS, SIRS and MODS.

The structural properties of proteins may be affected by divalent cations. For example, aggregation and/or precipitation of proteins, as well as oligomerization, may be induced by divalent cations. Aggregation of proteins can impact the ability to produce, purify, formulate and deliver a protein, for example, as a pharmaceutical product. Moreover, aggregation and/or oligomerization can impact the stability of a protein, for example, in storage. In some instances, a protein's stability can be enhanced if aggregated and/or precipitated prior to, or during storage.

FLINT and analogs thereof, for example, protease-resistant analog R218Q, aggregate and eventually precipitate from solution when exposed to divalent cation. For example, analog R218Q purified by IMAC chromatography and elution in 0.4M imidazole, precipitates from solution (See Example 6, *infra*). These observations suggest that FLINT and analogs thereof interact with divalent cations, such as Ni^{+2}, to cause aggregation and/or precipitation.

As FLINT analogs are potentially useful therapeutic proteins, their purification and formulation are important factors to be worked out on the path to development of a pharmaceutical product. While FLINT is known from prior disclosures (See e.g WO 98/30694 and WO 99/50413), its formulation has not been thoroughly investigated, nor has the impact of divalent cation on the aggregation and/or oligomerization of the protein and its protease-resistant analogs been sufficiently investigated for purposes of realizing the full therapeutic and pharmaceutical utility.

The present invention relates to a method for eliminating aggregation and/or precipitation of protease-resistant FLINT analog(s), useful in purifying FLINT analogs comprising the removal of divalent cation from a solution or other medium comprising FLINT analog(s).

The invention relates further to the purification of FLINT analogs from a solution of one or more of said FLINT analogs, by immobilized metal ion affinity (IMAC) chromatography, comprising removal of divalent cation from said solution.

The invention relates further to a composition comprising a protease-resistant FLINT analog and a divalent metal cation.

The invention relates further to a method for producing a composition comprising a protease-resistant FLINT analog, in association with a divalent cation.

The present invention relates further to a pharmacuetical formulation comprising a protease-resistant FLINT analog, in association with a divalent metal cation, and with one or more pharmaceutically acceptable carriers, diluents, or excipients.

Accordingly, the present invention provides a FLINT analog-divalent cation complex, which comprises a FLINT analog complexed with a divalent metal cation, pharmaceutical formulations thereof, and methods for using such pharmaceutical formulations for the treatment and/or prevention of disorders that may be associated with the binding of Fas to FasL, and/or LIGHT to the LTβR and/or TR2/HVEM receptors.

Compounds such as protease-resistant FLINT which inhibit the binding of Fas to Fas Ligand or LIGHT to LTβR and/or TR2/HVEM receptors can be used to treat or prevent

diseases or conditions associated with these binding interactions.

The present invention provides conditions under which potency and/or stability of protease-resistant FLINT analogs may be significantly enhanced. Thus, effective pharmacological treatment using protease-resistant FLINT may be achieved at lower doses thereby potentially abrogating toxic or other undesirable side effects. Accordingly, the present invention provides a protein-cation complex, which comprises a protease-resistant FLINT analog, or FLINT fusion protein comprising a protease-resistant FLINT analog complexed with a divalent metal cation.

Summary of the Invention

The invention provides a composition comprising a protease-resistant FLINT analog or fusion protein thereof complexed with a divalent metal cation. The invention additionally provides parenteral pharmaceutical formulations comprising the FLINT-cation compounds and methods of using such compounds for treating or preventing diseases and disorders, e.g. those that may be associated with the binding of Fas to FasL, and/or LIGHT to LTβR and/or TR2/HVEM receptors. The invention further provides a process of preparing such compounds, which comprises combining a protease-resistant FLINT analog or fusion protein comprising a FLINT analog and a divalent metal cation in an aqueous solution at a pH of about 4.5 to 9.0.

Detailed Description and Preferred Embodiments

For purposes of the present invention, as disclosed and claimed herein, the following terms and abbreviations are defined as follows:

The term "aggregate" or "aggregation" refers to a non-covalent association of protein or peptide molecules including monomers, subunits, and fragments thereof, that may lead to precipitation of said molecules.

"FLINT protein analog," "FLINT analog," or "analog" refers to a protein derivative of mature FLINT (SEQ ID NO:1) or native FLINT (SEQ ID NO:2) comprising one or more amino acid deletions, additions, substitutions or inversions of residues within SEQ ID NO:1 or SEQ ID NO:2 that comprise FLINT analogs that are resistant to proteolysis between positions 218 and 219 of SEQ ID NO:1 (alternatively, between positions 247 and 248 of SEQ ID NO:3). Also included in the term are FLINT fusion proteins.

"FLINT" is used herein to encompass FLINT analogs and FLINT fusion proteins.

FLINT analogs may comprise multiple changes to native FLINT, including e.g. one or more changes that effect glycosylation in combination with one or more changes that impart protease resistance at position 218 of SEQ ID NO:1. An specific example from this genre includes the analog RDDSR (R34N, D36T, D194N, S196T, R218Q).

The term "fusion protein" or "FLINT fusion protein" as used herein refers to a FLINT protein or analog thereof wherein said protein or analog is fused to a heterologous protein or peptide including a peptide tag useful in purification, e.g. a His-tag.

The term "negatively charged group" or "negatively charged amino acid" refers to Asp or Glu.

The term "positively charge group" or "positively charged amino acid" refers to His, Arg, or Lys.

The term "polar uncharged" or "polar uncharged amino acid" refers to Cys, Thr, Ser, Gly, Asn, Gln, and Tyr.

The term "nonpolar" or "nonpolar amino acid" refers to Ala, Pro, Met, Leu, Ile, Val, Phe, or Trp.

The term "naturally-occurring amino acid" refers to any of the 20 L-amino acids that are found in proteins.

"Treating" as used herein, describes the management and care of a patient for the purpose of combating the disease, condition, or disorder and includes the administration of a protein of the present invention to prevent the onset of the symptoms or complications, alleviating the symptoms or complications, or eliminating the disease, condition, or disorder.

"Isotonicity agent" refers to an agent that is physiologically tolerated and embarks a suitable tonicity to the formulation to prevent the net flow of water across the cell membrane. Compounds, such as glycerin, are commonly used for such purposes at known concentrations. Other possible isotonicity agents include salts, e.g., NaCl, dextrose, and lactose.

The term "oligomer" or "oligomerization" refers to a specific interaction of more than one protein subunit in non-covalent or covalent fashion. Examples of specific oligomers would include dimers, trimers, tetramers, etc. As used herein the term refers to association of one or more FLINT analogs including association of identical or non-identical subunits such as, for example, non-identical FLINT analogs in association. The process of oligomerization lies on a continuum with the process of

aggregation, the latter representing non-specific interactions, that in the extreme, lead to precipitation.

"Physiologically tolerated buffer" refers to buffers including TRIS, sodium acetate, sodium phosphate, or sodium citrate. The selection and concentration of buffer is known in the art.

"Pharmaceutically acceptable preservative" refers to a multi-use parenteral formulation that meets guidelines for preservative effectiveness to be a commercially viable product. Pharmaceutically acceptable preservatives known in the art as being acceptable in parenteral formulations include: phenol, m-cresol, benzyl alcohol, methylparaben, chlorobutanol, p-cresol, phenylmercuric nitrate, thimerosal and various mixtures thereof. Other preservatives may be found, e.g., in Wallhauser, K. H., Develop. Biol. Standard 24, 9-28 (Basel, S. Krager, 1974). The concentration necessary to achieve preservative effectiveness is dependent upon the preservative used and the conditions of the formulation.

The term "protease-resistant" or "resistant" refers herein to a FLINT analog that differs from FLINT by one or more amino acid substitutions, deletions, inversions, additions, or changes in glycosylation sites or patterns as described in PCT/US 00/06418. Preferably these changes occur in the region from about position 214 through position 222 of SEQ ID NO:1. The term "protease-resistant" contemplates degrees of resistance to proteolysis at position 218. The degree to which an analog is refractory to proteolysis at this position may vary from complete resistance to partial resistance. All such embodiments are intended to be within the scope of the invention. Resistance is described herein in terms relative to the

sensitivity of FLINT to proteolysis *in vivo* or *in vitro*. For example, the resistance of an analog to a serine protease such as thrombin or trypsin, or other protease may be compared against the resistance shown by FLINT. It is preferred that a FLINT analog display a half-life at least 5% greater than FLINT, alternatively at least 10%, 20%, 30%, 40%, or between 50% to 100% greater than wild type FLINT, as determined by the relative amounts of full length molecule compared with smaller digestion products (e.g. 1-218 and 219-271 of SEQ ID NO:1). Most preferably, a resistant analog possesses a half-life that is from about 1-fold to 2-fold greater than FLINT to about 100-fold or greater than FLINT.

As used herein "half-life" refers to the time required for approximately half the FLINT or analog molecules to be proteolytically cleaved between positions 218 and 219 of SEQ ID NO:1, or comparable sequence of an analog, as determined by any suitable means, for example, a scan of a PAGE profile of digestion products.

As noted above, the invention provides a compound comprising a FLINT analog complexed with a divalent metal cation.

In one embodiment, the invention relates to a FLINT analog comprising one or more amino acid substitution(s) in the region 214-222 of SEQ ID NO:1, and/or amino acids 243-251 of SEQ ID NO:3 said analog being resistant to proteolysis at position 218 of SEQ ID NO:1.

In another embodiment, the invention relates to a FLINT analog comprising an amino acid substitution(s) in the region comprising amino acids 214 - 222 of SEQ ID NO:1, selected from the group consisting of:

a. Gly at position 214 is replaced by any naturally occurring amino acid other than Gly;
b. Pro at position 215 is replaced by any naturally occurring amino acid other than Pro;
c. Thr at position 216 is replaced by any naturally occurring amino acid other than Thr;
d. Pro at position 217 is replaced by any naturally occurring amino acid other than Pro;
e. Arg at position 218 is replaced by any naturally occurring amino acid other than Arg;
f. Ala at position 219 is replaced by any naturally occurring amino acid other than Ala; and
g. Gly at position 222 is replaced by any naturally occurring amino acid other than Gly.

In another embodiment, the invention relates to a FLINT analog comprising an amino acid substitution in the region comprising amino acids 214 - 222 of SEQ ID NO:1, selected from the group consisting of:

a. Gly at position 214 is replaced by a positively charged amino acid that is not Gly;
b. Pro at position 215 is replaced by a positively charged amino acid that is not Pro;
c. Thr at position 216 is replaced by a positively charged amino acid that is not Thr;
d. Pro at position 217 is replaced by a positively charged amino acid that is not Pro;
e. Arg at position 218 is replaced by a positively charged amino acid that is not Arg;
f. Ala at position 219 is replaced by a positively charged amino acid that is not Ala;
g. Gly at position 222 is replaced by a positively charged amino acid that is not Gly.

In another embodiment, the invention relates to a FLINT analog comprising an amino acid substitution in the region comprising amino acids 214 - 222 of SEQ ID NO:1, selected from the group consisting of:

a. Gly at position 214 is replaced by a negatively charged amino acid that is not Gly;
b. Pro at position 215 is replaced by a negatively charged amino acid that is not Pro;
c. Thr at position 216 is replaced by a negatively charged amino acid that is not Thr;
d. Pro at position 217 is replaced by a negatively charged amino acid that is not Pro;
e. Arg at position 218 is replaced by a negatively charged amino acid that is not Arg;
f. Ala at position 219 is replaced by a negatively charged amino acid that is not Ala;
g. Gly at position 222 is replaced by a negatively charged amino acid that is not Gly.

In another embodiment, the invention relates to a FLINT analog comprising an amino acid substitution in the region comprising amino acids 214 - 222 of SEQ ID NO:1, selected from the group consisting of:

a. Gly at position 214 is replaced by a polar uncharged amino acid that is not Gly;
b. Pro at position 215 is replaced by a polar uncharged amino acid that is not Pro;
c. Thr at position 216 is replaced by a polar uncharged amino acid that is not Thr;
d. Pro at position 217 is replaced by a polar uncharged amino acid that is not Pro;
e. Arg at position 218 is replaced by a polar uncharged amino acid that is not Arg;

f. Ala at position 219 is replaced by a polar uncharged amino acid that is not Ala;

g. Gly at position 222 is replaced by a polar uncharged amino acid that is not Gly.

In another embodiment, the invention relates to a FLINT analog comprising an amino acid substitution in the region comprising amino acids 214 - 222 of SEQ ID NO:1, selected from the group consisting of:

a. Gly at position 214 is replaced by a nonpolar amino acid that is not Gly;

b. Pro at position 215 is replaced by a nonpolar amino acid that is not Pro;

c. Thr at position 216 is replaced by a nonpolar amino acid that is not Thr;

d. Pro at position 217 is replaced by a nonpolar amino acid that is not Pro;

e. Arg at position 218 is replaced by a nonpolar amino acid that is not Arg;

f. Ala at position 219 is replaced by a nonpolar amino acid that is not Ala;

g. Gly at position 222 is replaced by a nonpolar amino acid that is not Gly.

In another embodiment, the invention relates to a FLINT analog comprising an amino acid substitution in the region comprising amino acids 214 - 222 of SEQ ID NO:1, selected from the group consisting of:

a. Arg at position 218 is replaced by Gln;

b. Arg at position 218 is replaced by Glu;

c. Thr at position 216 is replaced by Pro;

d. Arg at position 218 is replaced by Ala;

e. Arg at position 218 is replaced by Gly;

f. Arg at position 218 is replaced by Ser;

g. Arg at position 218 is replaced by Val

h. Arg at position 218 is replaced by Tyr;

i. Pro at position 217 is replaced by Tyr;

j. Thr at position 216 is replaced by Pro and Arg at position 218 is replaced by Gln;

k. Arg at position 34 is replaced by Asn, Asp at position 36 is replaced by Thr, and Arg at position 218 is replaced by Gln, Glu, Ala, Gly, Ser, Val, or Tyr;

l. Arg at position 34 is replaced by Asn, Asp at position 36 is replaced by Thr, and Arg at position 218 is replaced by Gln;

m. Arg at position 34 is replaced by Asn, Asp at position 36 is replaced by Thr, Asp at position 194 is replaced by Asn, Ser at position 196 is replaced by Thr, and Arg at position 218 is replaced by Gln, Glu, Ala, Gly, Ser, Val, or Tyr;

n. Arg at position 34 is replaced by Asn, Asp at position 36 is replaced by Thr, Asp at position 194 is replaced by Asn, Ser at position 196 is replaced by Thr, and Arg at position 218 is replaced by Gln.

Applicants have discovered that protease-resistant FLINT analogs undergo oligomeriztion and/or aggregation in the presence of divalent cations. In one aspect of the present invention, pharmaceutical compositions of these FLINT analogs and divalent cation provide depot formulations for therapeutic use. In another aspect, oligomerization and/or aggregation of FLINT analog can be reduced, prevented, or reversed by removal of divalent cation from said protein. In this aspect, the

invention relates to a process or method for purifying a FLINT analog and for maintaining a FLINT analog in solution.

The presently claimed compositions comprise FLINT analogs complexed with a divalent metal cation. A divalent metal cation includes, for example, Zn^{+2}, Mn^{+2}, Fe^{+2}, Co^{+2}, Cd^{+2}, Ca^{+2}, Ni^{+2} and the like. A combination of two or more divalent metal cations is operable; however the preferred compositions comprise a single species of metal cation, most preferably Zn^{++}. Preferably, the divalent metal cation is in excess; however, the molar ratio of at least one molecule of a divalent metal cation for each ten molecules of FLINT analog is operable. Preferably, the compositions comprise from 1 to 100 divalent metal cations per molecule of FLINT analog. The compositions may be amorphous or crystalline solids.

Appropriate forms of metal cations are any form of a divalent metal cation that is available to form a complex with a molecule of FLINT analog of the present invention. The metal cation may be added in solid form or it may be added as a solution. Several different cationic salts can be used in the present invention. Representative examples of metal salts include the acetate, bromide, chloride, fluoride, iodide and sulfate salt forms. The skilled artisan will recognize that there are many other metal salts which also might be used in the production of the compounds of the present invention. Preferably, zinc acetate or zinc chloride is used to create the zinc-FLINT analog compounds of the present invention. Most preferably, the divalent metal cationic salt is zinc chloride.

Generally, the claimed compounds are prepared by techniques known in the art. For example, convenient preparation is to combine FLINT analog with the desired divalent metal cation in an aqueous solution at a pH of about 4.5-9.0, preferably about pH 5.5-8, most preferably, pH 6.5-7.6. The claimed compound precipitates from the solution as a crystalline or amorphous solid. Significantly, the compound is easily isolated and purified by conventional separation techniques appreciated in the art including filtration and centrifugation. Significantly, the protein-metal cation complex is stable and may be conveniently stored as a solid or as an aqueous suspension.

The present invention further provides a pharmaceutical formulation comprising a compound of the present invention and water. The concentration of the FLINT analog in the formulation is about 0.1 mg/mL to about 100 mg/mL; preferably about 0.5 mg/mL to about 50.0 mg/mL; most preferably, about 5.0 mg/mL.

The formulation preferably comprises a pharmaceutically acceptable preservative at a concentration necessary to maintain preservative effectiveness. The relative amounts of preservative necessary to maintain preservative effectiveness varies with the preservative used. Generally, the amount necessary can be found in Wallhauser, K. H., Develop. Biol. Standard 24, 9-28 (Basel, S. Krager, 1974), herein incorporated by reference.

An isotonicity agent, preferably glycerin, may be added to the formulation. The concentration of the isotonicity agent is in the range known in the art for parenteral formulations, preferably about 16 mg/mL glycerin. The pH of the formulation may also be buffered

with a physiologically tolerated buffer. Acceptable physiologically tolerated buffers include TRIS, sodium acetate, sodium phosphate, or sodium citrate. The selection and concentration of buffer is known in the art.

Other additives, such as a pharmaceutically acceptable excipients like Tween 20 (polyoxyethylene (20) sorbitan monolaurate), Tween 40 (polyoxyethylene (20) sorbitan monopalmitate), Tween 80 (polyoxyethylene (20) sorbitan monooleate), Pluronic F68 (polyoxyethylene polyoxypropylene block copolymers), BRIJ 35 (polyoxyethylene (23) lauryl ether), and PEG (polyethylene glycol) may optionally be added to the formulation to reduce aggregation.

The claimed pharmaceutical formulations are prepared in a manner known in the art, and are administered individually or in combination with other therapeutic agents. The formulations of the present invention can be prepared using conventional dissolution and mixing procedures. Preferably, the claimed formulations are prepared in an aqueous solution suitable for parenteral use. That is, a protein solution is prepared by mixing water for injection, buffer, and a preservative. Divalent metal cations are added to a total cation concentration of about 0.001 to 5.0 mg/mL, preferably 0.05 to 1.5 mg/mL. The pH of the solution may be adjusted to completely precipitate the FLINT analog-cation complex. The compound is easily resuspended before administration to the patient.

Parenteral daily doses of the compound are in the range from about 1 ng to about 10 mg per kg of body weight, although lower or higher dosages may be administered. The required dosage will be determined by the physician and will depend on the severity of the condition of the patient

and upon such criteria as the patient's height, weight, sex, age, and medical history.

Variations of this process would be recognized by one of ordinary skill in the art. For example, the order the components are added, if a the surfactant is used, the temperature, and pH at which the formulation is prepared may be optimized for the concentration and means of administration used.

The pH of the formulation is generally pH 4.5 to 9.0 and preferably 5.5 to 8.0, most preferably 6.5 to 7.6; although more acidic pH wherein a portion or all of the protein-metal cation complex is in solution is operable.

The formulations prepared in accordance with the present invention may be used in a syringe, injector, pumps or any other device recognized in the art for parenteral administration.

The proteins used in the present compounds can be prepared by any of a variety of recognized peptide synthesis techniques including classical (solution) methods, solid phase methods, semi synthetic methods, and more recent recombinant DNA methods. Recombinant methods are preferred if a high yield is desired. The basic steps in the recombinant production of protein include:

a) construction of a synthetic or semi-synthetic (or isolation from natural sources) DNA encoding the FLINT analog,

b) integrating the coding sequence into an expression vector in a manner suitable for the expression of the protein either alone or as a fusion protein,

c) transforming an appropriate eukaryotic or prokaryotic host cell with the expression vector, and

d) recovering and purifying the recombinantly produced protein.

A cDNA encoding native FLINT (SEQ ID NO:3) can provide a template from which to engineer specific mutations that result in a nucleic acid that encodes an analog of the invention. For example, FLINT cDNA is used as a template for introducing appropriate point mutations (i.e. construction of FLINT analog cDNAs). A suitable protocol is described in detail in "Current Protocols in Molecular Biology", volume 1, section 8.5.7 (John Wiley and Sons, Inc. publishers), incorporated herein by reference. Briefly, synthetic oligonucleotides are designed to incorporate one or more desired point mutation(s) at one end of an amplified fragment, e.g. at position 218 of SEQ ID NO:1. Following first strand PCR, the amplified fragments encompassing the mutation are annealed with each other and extended by mutually primed synthesis. Annealing is followed by a second PCR step utilizing 5' forward and 3' reverse end primers in which the entire mutagenized fragment gets amplified and is ready for subcloning into the appropriate vector.

Synthetic genes and nucleic acids can be constructed by techniques well known in the art. Owing to the degeneracy of the genetic code, the skilled artisan will recognize that multiple DNA sequences may be constructed which encode the desired proteins. Synthesis is achieved by recombinant DNA technology or by chemical synthesis, for example, see Brown, et al. (1979) Methods in

Enzymology, Academic Press, N.Y., Vol. 68, pgs. 109-151. A DNA sequence(s) encoding FLINT analogs can be generated using a conventional DNA synthesizing apparatus such as the Applied Biosystems Model 380A or 380B DNA synthesizers (commercially available from Applied Biosystems, Inc., 850 Lincoln Center Drive, Foster City, CA 94404). It may be desirable in some applications to modify the coding sequence of a FLINT analog so as to incorporate a convenient protease sensitive cleavage site, e.g., between the signal peptide and the structural protein facilitating the controlled excision of the signal peptide from the fusion protein construct.

A gene encoding FLINT analog(s) may also be created by using the polymerase chain reaction (PCR). The template can be a cDNA library, for example (commercially available from CLONETECH or STRATAGENE). Such methods are well known in the art, *c.f.* Maniatis, et al. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989), herein incorporated by reference.

Alternatively, a cDNA encoding native FLINT (SEQ ID NO:3) can provide a template from which to engineer specific mutations that result in a nucleic acid that encodes an analog of the invention. For example, FLINT cDNA is used as a template for introducing appropriate point mutations (i.e. construction of FLINT analog cDNAs). A suitable protocol is described in detail in "Current Protocols in Molecular Biology", volume 1, section 8.5.7 (John Wiley and Sons, Inc. publishers), incorporated herein by reference. Briefly, synthetic oligonucleotides are designed to incorporate one or more desired point mutation(s) at one end of an amplified fragment, e.g. at

position 218 of SEQ ID NO:1. Following first strand PCR, the amplified fragments encompassing the mutation are annealed with each other and extended by mutually primed synthesis. Annealing is followed by a second PCR step utilizing 5' forward and 3' reverse end primers in which the entire mutagenized fragment gets amplified and is ready for subcloning into the appropriate vector.

The constructed or isolated DNA sequences of the invention are useful for expressing FLINT analog. When the sequences comprise a fusion gene, the resulting product, if desired, can be treated enzymatically or chemically to release FLINT analog. A variety of suitable peptidases are known that cleave a polypeptide at specific sites, or digest the peptides from the amino or carboxy termini (e.g. diaminopeptidase). Furthermore, particular chemicals (e.g. cyanogen bromide) will cleave a polypeptide chain at specific sites. The skilled artisan will appreciate the modifications necessary to the amino acid sequence (and synthetic or semi-synthetic coding sequence if recombinant means are employed) to incorporate site-specific internal cleavage sites. See U.S. Patent No. 5,126,249; Carter P., Site Specific Proteolysis of Fusion Proteins, Ch. 13 in Protein Purification: From Molecular Mechanisms to Large Scale Processes, American Chemical Soc., Washington, D.C. (1990).

Construction of suitable vectors containing the desired coding and control sequences employ standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to form the plasmids required.

In general, plasmid vectors containing promoters and control sequences which are derived from species

compatible with the host cell are used with these hosts. The vector ordinarily carries a replication origin and one or more sequences for selection of transformed cells.

The desired coding sequence is inserted into an expression vector in the proper orientation to be transcribed from a promoter and ribosome binding site, both of which should be functional in the host cell in which the protein is to be expressed.

In general, procaryotes are used for cloning of DNA sequences in constructing the vectors useful in the invention. For example, E. coli K12 strain 294 (ATCC No. 31446) is particularly useful. Other microbial strains which may be used include E. coli B and E. coli X1776 (ATCC No. 31537). These examples are illustrative rather than limiting.

The DNA molecules may also be recombinantly expressed in eukaryotic expression systems. Preferred promoters controlling transcription in mammalian host cells may be obtained from various sources, for example, the genomes of viruses such as: polyoma, Simian Virus 40 (SV40), adenovirus, retroviruses, hepatitis-B virus and most preferably cytomegalovirus, or from heterologous mammalian promoters, e.g. •-actin promoter. The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment which also contains the SV40 viral origin of replication. Fiers, et al., Nature, 273:113 (1978). The entire SV40 genome may be obtained from plasmid pBRSV, ATCC 45019. The immediate early promoter of the human cytomegalovirus may be obtained from plasmid pCMBb (ATCC 77177). Of course, promoters from the host cell or related species also are useful herein.

Transcription of the DNA by higher eucaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about 10-300 bp, that act on a promoter to increase its transcription. Enhancers are relatively oriented and positioned independently and have been found 5' (Laimins, L. et al., PNAS 78:993 (1981)) and 3' (Lusky, M. L., et al., Mol. Cell Bio. 3:1108 (1983)) to the transcription unit, within an intron (Banerji, J. L. et al., Cell 33:729 (1983)) as well as within the coding sequence itself (Osborne, T. F., et al., Mol. Cell Bio. 4:1293 (1984)). Many enhancer sequences are now known from mammalian genes (globin, RSV, SV40, EMC, elastase, albumin, alpha-fetoprotein and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 late enhancer, the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription which may affect mRNA expression. These regions are transcribed as polyadenylated segments in the untranslated portion of the mRNA encoding protein. The 3' untranslated regions also include transcription termination sites.

Expression vectors may contain a selectable marker. Examples of suitable selectable markers for mammalian cells are dihydrofolate reductase (DHFR, which may be derived from the BglII/HindIII restriction fragment of pJOD-10 [ATCC 68815]), thymidine kinase (herpes simplex virus thymidine kinase is contained on the BamHI fragment

of vP-5 clone [ATCC 2028]) or neomycin (G418) resistance genes (obtainable from pNN414 yeast artificial chromosome vector [ATCC 37682]). When such selectable markers are successfully transferred into a mammalian host cell, the transfected mammalian host cell can survive if placed under selective pressure. There are two widely used distinct categories of selective regimes. The first category is based on a cell's metabolism and the use of a mutant cell line which lacks the ability to grow without a supplemented media. Two examples are: CHO DHFR$^-$ cells (ATCC CRL-9096) and mouse LTK$^-$ cells (L-M(TK-) ATCC CCL-2.3). These cells lack the ability to grow without the addition of such nutrients as thymidine or hypoxanthine. Because these cells lack certain genes necessary for a complete nucleotide synthesis pathway, they cannot survive unless the missing nucleotides are provided in a supplemented media. An alternative to supplementing the media is to introduce an intact DHFR or TK gene into cells lacking the respective genes, thus altering their growth requirements. Individual cells which were not transformed with the DHFR or TK gene will not be capable of survival in nonsupplemented media.

A suitable vector for eucaryotic expression is pRc/CMV. pRc/CMV is commercially available from Invitrogen Corporation, 3985 Sorrento Valley Blvd., San Diego, CA 92121. To confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform E. coli K12 strain DH10B (ATCC 31446) and successful transformants selected by antibiotic resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction and/or sequence by the method of Messing, et al., Nucleic Acids Res. 9:309 (1981).

Host cells may be transformed with the expression vectors of this invention and cultured in conventional nutrient media modified as is appropriate for inducing promoters, selecting transformants or amplifying genes. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan. The techniques of transforming cells with the aforementioned vectors are well known in the art and may be found in such general references as Maniatis, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989), or Current Protocols in Molecular Biology (1989) and supplements.

Suitable host cells for expressing the vectors encoding the claimed proteins in higher eucaryotes include: African green monkey kidney line cell line transformed by SV40 (COS-7, ATCC CRL-1651); transformed human primary embryonal kidney cell line 293,(Graham, F. L. et al., J. Gen Virol. 36:59-72 (1977), Virology 77:319-329, Virology 86:10-21); baby hamster kidney cells (BHK-21(C-13), ATCC CCL-10, Virology 16:147 (1962)); Chinese hamster ovary cells CHO-DHFR$^-$ (ATCC CRL-9096), mouse Sertoli cells (TM4, ATCC CRL-1715, Biol. Reprod. 23:243-250 (1980)); African green monkey kidney cells (VERO 76, ATCC CRL-1587); human cervical epitheloid carcinoma cells (HeLa, ATCC CCL-2); canine kidney cells (MDCK, ATCC CCL-34); buffalo rat liver cells (BRL 3A, ATCC CRL-1442); human diploid lung cells (WI-38, ATCC CCL-75); human hepatocellular carcinoma cells (Hep G2, ATCC HB-8065);and mouse mammary tumor cells (MMT 060562, ATCC CCL51).

In addition, unicellular eukaryotes such as yeast may also be used. Saccharomyces cerevisiae, or common baker's yeast is the most commonly used eukaryotic microorganism, although a number of other strains are commonly available. For expression in Saccharomyces, the plasmid YRp7, for example, (ATCC-40053, Stinchcomb, et al., Nature 282:39 (1979); Kingsman et al., Gene 7:141 (1979); Tschemper et al., Gene 10:157 (1980)) is commonly used. This plasmid already contains the trp gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC no. 44076 or PEP4-1 (Jones, Genetics 85:12 (1977)).

Suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (found on plasmid pAP12BD ATCC 53231 and described in U.S. Patent No. 4,935,350, June 19, 1990) or other glycolytic enzymes such as enolase (found on plasmid pAC1 ATCC 39532), glyceraldehyde-3-phosphate dehydrogenase (derived from plasmid pHcGAPC1 ATCC 57090, 57091), zymomonas mobilis (United States Patent No. 5,000,000 issued March 19, 1991), hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which contain inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein (contained on plasmid vector pCL28XhoLHBPV ATCC 39475, United States Patent No. 4,840,896), glyceraldehyde 3-phosphate dehydrogenase, and

enzymes responsible for maltose and galactose (GAL1 found on plasmid pRY121 ATCC 37658) utilization. Suitable vectors and promoters for use in yeast expression are further described in R. Hitzeman et al., European Patent Publication No. 73,657A. Yeast enhancers such as the UAS Gal from Saccharomyces cerevisiae (found in conjunction with the CYC1 promoter on plasmid YEpsec--hI1beta ATCC 67024), also are advantageously used with yeast promoters.

The following examples are provided to further illustrate the preparation of the formuations of the invention. The scope of the invention is not construed as merely consisting of the following examples.

EXAMPLE 1

Preparation of FLINT analog-Zinc Formulations

About 20 mg of a FLINT analog in which the arginine residue at position 218 of SEQ ID NO:1 is replaced by glutamine (hereinafter referred to as "R218Q") is completely dissolved in 32 mL of an aqueous solution containing 16 mg/mL glycerin and 2 mg/mL phenol and passed through a sterile 0.2 μ filter. An aqueous solution containing 100 mg/mL of zinc in water is prepared from zinc chloride. Dilutions are made to prepare 10 mg/mL zinc and 1 mg/mL zinc solutions. Five 6-mL aliquots of the R218Q solution are mixed with the zinc solutions as shown in Table I:

Table I

Sample	mL of 1 mg/mL zinc added	ML of 10 mg/mL zinc added	ML of 100 mg/mL zinc added	ml of H_2O added	Total mg/mL zinc concentration
A	0	0	0	100	0
B	17	0	0	83	0.0027
C	0	33	0	67	0.054
D	0	0	19	81	0.30
E	0	0	92	8	1.50

Each formulation is adjusted to pH 7.48 ± 0.03 using small volumes of 2N and 5N sodium hydroxide and stored at 4°C. Sample A is completely clear while samples B through E are cloudy suspensions.

EXAMPLE 2

Analysis of Zinc Formulations

Size-exclusion chromatography is performed on the centrifuged supernatants of Samples A through E of Example 1. For these analyses, 100 uL of the supernatants are injected onto an analytical Superdex-75® (3.2/30, Pharmacia) column equilibrated in PBS (Dulbecco's Phosphate-Buffered Saline, GibcoBRL). The column is eluted at ambient temperature at 0.5 mL/min and the protein in the eluant monitored at 214 nm.

EXAMPLE 3

Biological Activity of the Zinc Formulations

A FLINT analog bioassay measuring cell survival (i.e. prevention of apoptosis) is performed in a 96 well plate format with reactions of 100 µl/well. 25 µl of Jurkat cells

($5X10^4$ cells/well) is mixed with 25 µl of recombinant human FasL (final concentration 150ng/ml) and 50 µl of FLINT analog in Example 1. Cells are incubated at 37°C overnight. Twenty µl of MTS tetrazolium compound (U.S. Pat. No. 5,185,450 assigned to the Univ. of South Florida and exclusively licensed to Promega Corporation, Madison, WI) is added to each well and the incubation carried out for 2h at 37°C. Absorbance at 490 nm is recorded using a plate reader.

EXAMPLE 4

Large Scale FLINT Analog R218Q Polypeptide Purification

Large scale production of a FLINT analog (containing a 6 histidine tag) was performed by growing stable pools in several roller bottles. After reaching confluency, cells were further incubated in serum-free medium for 5 to 7 days to secrete maximum amount of a FLINT analog into the medium. Media containing FLINT analog was adjusted to 0.1 % CHAPS concentrated in an Amicon ProFlux M12 tangential filtration system to 350 ml using an Amicon S3Y10 UF membrane. The concentrated media was passed over IMAC (Immobilized Metal-Affinity Chromatography (Pharmacia, 5 to 20 ml column) at a flow rate of 1 ml/min. The column was washed with buffer A (PBS (1 mM potassium phosphate, 3 mM sodium phosphate), 0.5 M NaCl, pH 7.4) until the absorbence returned to baseline and the bound polypeptides were eluted with a linear gradient from 0.025 M to 0.5 M Imidazol (in buffer A) developed over 60 min. Fractions containing FLINT analog were pooled and EDTA is added to a final concentration of 50 mM EDTA. The pooled fractions containing FLINT analog are concentrated using an Ultrafree centrifugal filter unit (Millipore, 10 kDa molecular weight cut-off) to 2 ml. This material was passed over a Superdex 75 (Pharmacia, 16/60) sizing column equilibrated with PBS, 0.5 M NaCl, pH 7.4, at a flow rate of 1 ml/min. Fractions containing FLINT analog

were analyzed by SDS-PAGE. The N-terminal sequence of FLINT analog was confirmed on the purified polypeptide.

EXAMPLE 5

Interaction of R218Q His-tagged FLINT With Ni^{2+} Demonstrated by Analytical SEC

FLINT analog R218Q His-tagged (50 ul, 0.1 mg/ml) was incubated with $NiCl_2$ (final concentration of 1 mM) or with $NiCl_2$ and EDTA (both at a final concentration of 1 mM) for at 4 °C for 2 hours. As a control, R218Q His-tagged FLINT was treated without the addition of $NiCl_2$ or EDTA. After the incubation, the samples were centrifuged in an Eppendorf centrifuge at maximum speed for 5 min. 20 µl of each sample was injected on to an analytical Superdex 75 column and eluted from this column at a flow rate of 70 µl/min in PBS, 0.5 M NaCl, pH 7.4. The results are summarized in Figure 1.

EXAMPLE 6

Effect of Divalent Cation on FLINT and Analogs

FLINT and FLINT analogs were purified from either AV12 or 293 cell lines. Protein samples were stored in PBS at pH 7.4, 0.5 M NaCl, and 10% glycerol. The effect of divalent cations, such as Ni^{2+}, Zn^{2+} and Ca^{2+}, was investigated using intrinsic tryptophan fluorescence intensity and fluorescence anisotropy. Since fluorescence anisotropy is very sensitive to the rotational correlation time of the molecule, the change in the value of anisotropy reflects change in the association of FLINT molecules upon addition of divalent cations.

Concentrations of FLINT or FLINT analogs were measured on an AVIV model 14DS spectrometer. Spectra were collected

from 400 nm to 260 nm at 1-nm bandwidth and were corrected for the solvent and scatter using data obtained between 360 nm to 320 nm by the AVIV computer program Loggen. The peak absorbance at about 280 nm was divided by 0.786 mg^{-1} cm^{-1} to determine the concentration of the protein in a 1-cm pathlength cell. 5 mM $NiCl_2$ or $ZnCl_2$ or $CaCl_2$ stock solution was made by dissolving in H_2O the appropriate amount of solid $NiCl_2$, $ZnCl2_2$, or $CaCl_2$.

Tryptophan fluorescence intensity and fluorescence anisotropy were measured using an ISS PCI photon counting spectrofluorometer. A protein solution of about 0.1 mg/ml concentration was excited at 295 nm and the total intensity of fluorescence and fluorescence anisotropy was recorded using a 335 nm cutoff filter in a cell of 5 mm x 10 mm pathlength with a 8 nm excitation bandwidth. A small aliquot of 5 mM $NiCl_2$, $ZnCl_2$, or $CaCl_2$ stock was added to the protein sample in the cell to adjust the concentration of divalent cation concentration. The sample was then mixed by inverting the cuvette after each addition of divalent metal . The fluorescence signal intensity and anisotropy were determined as a function of divalent ion concentration.

The fluorescence intensity and anisotropy data obtained on FLINT as a function of $NiCl_2$ or $ZnCl_2$ is shown in Table I. Addition of either $NiCl_2$ or $ZnCl_2$ decreased the fluorescence intensity and increased the anisotropy, indicating an association of FLINT molecules. The association of FLINT molecules upon addition of $ZnCl_2$ is reversible by addition of 2 mM EDTA, as indicated by the decrease of anisotropy to the initial anisotropy value in the absence of $ZnCl_2$.

Table I. Typtophan fluorescence intensity and anisotropy of FLINT as a function of $NiCl_2$ or $ZnCl_2$ concentration.

[NiCl2] uM	Intensity	Anisotropy	[ZnCl2] uM	Anisotropy
0	669978	0.1357	0	0.1344
5	648081	0.14	5	0.1304
10	628453	0.1368	10	0.1273
20	578457	0.1396	20	0.1342
40	548057	0.1416	40	0.1462
80	514699	0.1461	80	0.1724
100	457960	0.1527	100	0.1884
200	443962	0.1578	200	0.2172
400	361070	0.1671	400	0.2436
+ 2 mM EDTA	442029	0.1528	2 mM EDTA	0.1338

The effect of $NiCl_2$ and $ZnCl_2$ on His-tagged R218Q FLINT was also investigated. In contrast to FLINT, addition of small concentration of $NiCl_2$ or $ZnCl_2$ causes precipitation of His-tagged R218Q, leading to the rapid increase of fluorescence anisotropy, as shown in Table II. The precipitation caused by $ZnCl_2$ can be readily reversed by addition of 2 mM EDTA. However, the precipitation by $NiCl_2$ can only be reversed very slowly.

Table II. Fluorescence anisotropy of His-tagged R218Q FLINT as a function of $NiCl_2$ or $ZnCl_2$ concentration.

[NiCl2] uM	Anisotropy	[ZnCl2] uM	Anisotropy
0	0.1351	0	0.1299
5	0.1985	5	0.1360
10	0.214	10	0.2293
		+ 2 mM EDTA	0.1306

His-tagged analog RDDSR (i.e. R34N/D36T/D194N/S196T/R218Q) FLINT was purified from transiently-transfected 293EBNA cell line. This analog contains two additional putative asparagine-linked glycosylation sites at Asn34 and Asn194. Fluorescence intensity and anisotropy as a function of divalent cation concentration are shown in Table III. In comparison to His-tagged R218Q, the hyperglycosylated His-tagged RDDSR is much less sensitive to $NiCl_2$. Addition of $NiCl_2$ up to 400 uM did not cause visible precipitation of protein. However, $ZnCl_2$ does cause the protein to precipitate, although to a lesser degree compared to His-tagged R218Q FLINT. The precipitated sample dissolved rapidly with addition of 1 mM EDTA and the anisotropy returned to the initial value in the absence of $ZnCl_2$. All three cations, Ni^{2+}, Zn^{2+}, and Ca^{2+}, appear to bind the His-tagged RDDSR FLINT analog, as suggested by the decrease of tryptophan fluorescence intensity as the concentrations of these cations were increased.

Table III. Fluorescence intensity and anisotropy of His-tagged RDDSR FLINT analog as a function of $NICl_2$, $ZnCl_2$, and $CaCl_2$ [a]

[$NiCl_2$] uM	Intensity	Anisotropy	[$ZnCl_2$] uM	Intensity	Anisotropy	[CaCl2] uM	Intensity	Anisotropy
0	791325	0.1246	0	881332	0.1446	0	959433	0.1356
5	735267	0.1322	5	879859	0.1326	5	940566	0.1337
10	699807	0.1334	10	847808	0.1369	10	946440	0.1358
20	682581	0.1292	20	821780	0.1523	20	937559	0.1333

40	685818	0.1349	40	813852	0.1860	40	872933	0.1268
80	667912	0.1379	80	780568	0.2137	80	849602	0.1331
100	645837	0.1368	+ 1mM EDTA	739379	0.1269	100	805338	0.1321
200	635790	0.1378				200	778321	0.1369
400	607711	0.1375				400	708589	0.1359

[a] Titration of $ZnCl_2$ was performed in 20 mM Tris, 150 mM NaCl at pH 7.4.

These examples show that divalent cations, such as Ni^{2+}, Zn^{2+}, and Ca^{2+}, interact with FLINT and FLINT analogs. The effect of these cations on the protein appears to be dependent on the nature of the analog. Both Ni^{2+} and Zn^{2+} induce association of FLINT molecules. Zn^{2+} causes reversible precipitation and can be used in the purification of His-tagged FLINT and analogs.

EXAMPLE 7

Treatment of Acute Liver Failure by a Complex of Divalent Cation and FLINT R218Q

Using mice, a model of liver damage is induced using a modification of the methods set out in Tsuji H., et al, 1997, Infection and Immunity, 65(5):1892-1898. FLINT R218Q is made according to Example 4. Briefly, BALB/c mice (Harlan) per each experimental group are given intravenous injections (the lateral tail vein) of 6 mg of D(+)-Galactosamine (Sigma, 39F-0539) in 100 µl of PBS (GIBCO-BRL) and 3 µg of Lipopolysaccharide B *E.coli* 026:B6 (LPS) (Difco, 3920-25-2) in 100 µl of PBS. The LPS is

administered, via i.v. injection, 5 minutes after the galactosamine, which was administered i.v. After LPS challenge, the animals are injected intraperitoneally with FLINT R218Q (200 μg), or R218Q complexed with Zinc (as in Example 1). The survival rates of the mice are determined 24 and 48 hours after LPS injection.

It is expected that the Zinc-FLINT R218Q formulation would be more potent than non-divalent formulated FLINT R218Q.

EXAMPLE 8

Treatment of Cerebral Ishcemia by a Complex of Divalent Cation and FLINT R218Q

Adult male gerbils (70 to 80 g body weight, Charles River Laboratories, Wilmington, MA) are anesthetized by i.p. injections of sodium pentobarbital (Nembutal) 40 mg/kg, and additional i.p. injections of 10 mg/kg when necessary to maintain a surgical plane of anesthesia. Animals are placed on a thermostatically controlled heating blanket to maintain body temperature at 37 °C. The ventral surface of the neck is exposed, the fur shaved, and the skin cleaned with 2% iodine solution.

After the pre-surgical preparation, a midline incision is made, and the skin opened. The sternohyoid muscles are divided to expose and isolate the common carotid arteries (CCA) for clamping. Sterilized aneurysm clips (blade with 0.15 mm, closing force ~10 gm) are secured by means of a sterilized clip applier on both left and right CCA for 5 minutes. The clamps are then removed and the patency of the arteries checked visually. The wound in the neck is closed by surgical suture.

Immediately following the cerebral ischemia procedure and while the gerbil is still unconscious, the fur on the dorsal surface of the head is shaved and the skin cleaned with 2% iodine solution. Under surgical anesthesia, the gerbil's head is secured in a stable position by means of a stereotaxic apparatus (SA) and a midline incision is made to expose the skull. At a position 1 mm lateral and 1 mm posterior to the bregma, as guided by the vernier scale of the SA, the skull is thinned by a dental drill equipped with a drill bit of 0.5 mm in diameter. The thinned area is punctured with a microsyringe equipped with a 27-guage blunt needle inserted 3 mm deep for a bolus injection of 5 ul (0.63 mg/ml)of FLINT in phosphate buffer saline (PBS) or zinc-FLINT R218Q complex of Example 1.

After the bolus injection, the syringe needle is exchanged for an infusion cannula [3 mm in length] of a brain infusion assembly connected to an Alzet osmotic pump (Alza Corp., Palo Alto, CA) which reservior is placed under the skin on the shoulder of the gerbil. The infusion cannula is anchored on the surface of the skull using dental cement. The wound is closed by surgical suture. The Alzet osmotic pump containing FLINT solution (0.63 mg/ml) or zinc-FLINT is delivered continuously at a rate of 1 ul/h for 3 days. Gerbils are allowed to survive for 5 days (the surgery day is taken as day zero).

On the fifth day of survival, the gerbils are sacrificed in a CO2 chamber. Thoracotomy is performed for transcardiac perfusion of saline for 3 minutes and formaldehyde for 2 minutes. The brains are removed for histological processing following a standard procedure commonly adapted in the field. Coronal sections are

obtained at approximately 1.7 mm posterior to the bregma. After staining with Cresyl violet, the sections are viewed under a microscope at 40x magnification for cell counter quantification of the intact hippocampal neurons along the dorsal CA1 regions (0.5 mm in length) of both hemispheres. Data are analyzed by Student t-Test and the Wilcox ranking test.

Previous results have shown that FLINT has a significant effect on neuronal survival compared to vehicle ($p=0.0039$ in t-Test; $p=0.0037$ in Wilcoxon Rank Sums). Zinc-FLINT R218Q complex is expected to have enhanced potency.

What is claimed is:

1. A composition comprising a divalent metal cation associated with a protease-resistant FLINT analog.

2. A composition as in claim 1 wherein said cation is selected from the group consisting of Zn^{+2}, Ca^{+2}, Ni^{+2}, Mn^{+2}, Fe^{+2}, Co^{+2}, and Cd^{+2}.

3. A composition of Claim 1, wherein the divalent metal cation is Zn^{+2}.

4. A composition of Claim 1, wherein the analog is resistant to proteolysis at position 218 of SEQ ID NO:1.

5. A composition of Claim 4 wherein arginine at position 218 of SEQ ID NO:1 is substituted by glutamine.

6. A pharmaceutical formulation comprising a composition of claim 5 in combination with one or more pharmaceutically acceptable carriers, diluents, or excipients.

7. A formulation of Claim 6, wherein the total cation concentration is 0.001 to 5.0 mg/mL.

8. A formulation of Claim 7, wherein the total cation concentration is 0.05 to 1.5 mg/mL.

9. A process for reducing aggregation of a protease-resistant FLINT analog molecule comprising the step of removing divalent metal cation.

10. A process as in claim 9 wherein said FLINT analog molecule is in solution.

11. A process as in claim 9 wherein said cation is removed by EDTA.

12. A method for inducing oligomerization of a protease-resistant FLINT analog molecule comprising the step of adding divalent metal cation.

13. A method for inducing aggregation of a protease-resistant FLINT analog molecule comprising the step of adding divalent metal cation until said analog precipitates.

Interaction of R218Q His-tagged FLINT Analog with Ni^{2+}

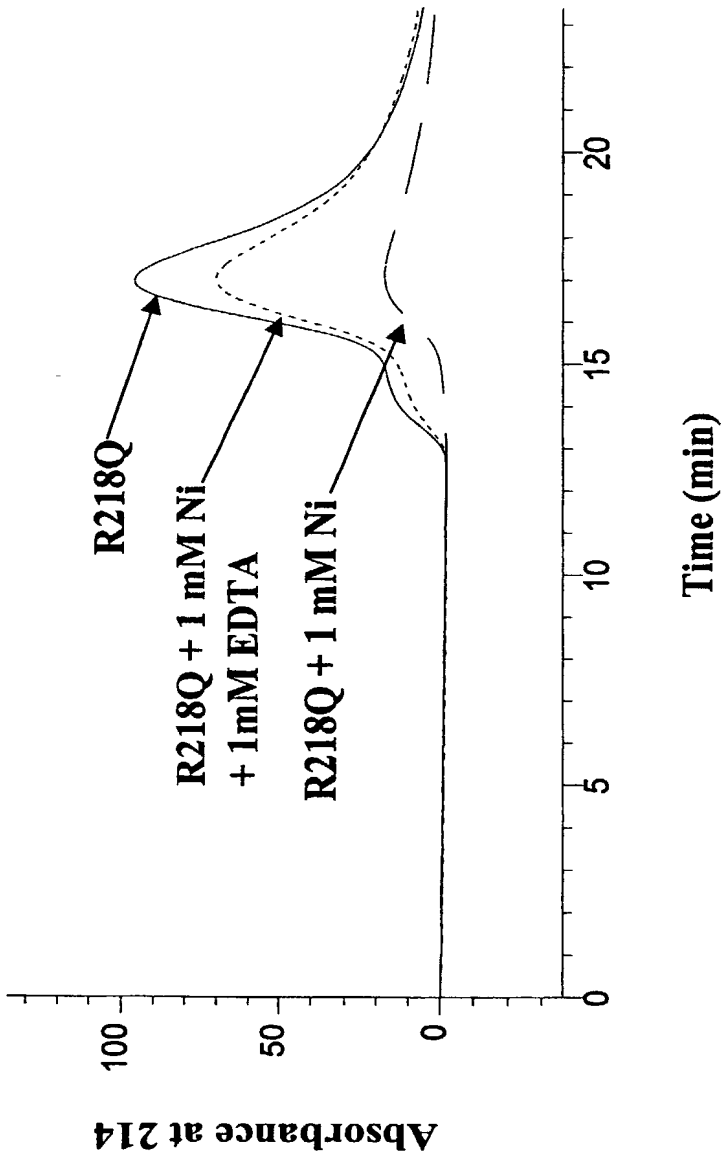

FIG. 1

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Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION

[X] Declaration Submitted with Initial Filing
[ ] Declaration Submitted after Initial Filing

Attorney Docket Number	**X-13268**
First Named Inventor	**Paul Robert Atkinson, et al.**
COMPLETE IF KNOWN	
Application Number	
Filing Date	
Group Art Unit	
Examiner Name	

As a below named Inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole Inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the Invention entitled:

FLINT ANALOG COMPOUNDS AND FORMULATIONS THEREOF

the specification of which
[ ] is attached hereto
OR
[X] was filed on (MM/DD/YYYY) 08/31/2000 as United States Application Number or PCT International Application Number PCT/US00/20806 and was amended on (MM/DD/YYYY) _____ (if applicable).

II hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37 Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or Inventor's certificate, or § 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached YES	Certified Copy Attached NO
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]
			[ ]	[ ]	[ ]

[ ] Additional foreign application numbers are listed on a supplemental priority sheet attached hereto:

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional applications(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)
60/153,433	09/10/1999

[ ] Additional provisional application numbers are listed on a supplemental priority sheet attached hereto.

Please type a plus sign (+) inside this box [+]

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DECLARATION

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s), or § 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application Number	PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number *(if applicable)*

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority sheet attached hereto.

As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

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Charles Joyner	30,466
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Michael J. Sayles	32,295
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Robert D. Titus	40,206
Robert C. Tucker	45,165
Tina M. Tucker	47,145
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Thomas D. Webster	39,872
Lawrence T. Welch	29,487
Alexander Wilson	45,782
Dan L. Wood	48,613

☐ Additional registered practitioner(s) named on a supplemental sheet attached hereto.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor: ☐ **A Petition has been filed for this unsigned inventor**

Given Name	Paul	**Middle Name**	Robert	**Family Name**	Atkinson	**Suffix e.g. Jr.**	
Inventor's Signature	Paul R. Atkinson					**Date**	1-30-02
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Post Office Address	SAME AS ABOVE						
City	Indianapolis	**State**	IN	**Zip**	46234	**Country**	US

[X] **Additional Inventors are being named on supplement sheet(s) attached hereto.**

Please type a plus sign (+) inside this box [+]

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DECLARATION

Name of Additional Joint Inventor, if any:													
						☐ A Petition has been filed for this unsigned inventor							
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City	Carmel	State	IN	Zip	46033	Country	US						

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						☐ A Petition has been filed for this unsigned inventor							
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Inventor's Signature										Date	1/29/02		
Residence: City	Fishers		State	IN		Country	US				Citizenship	US	
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Post Office Address	SAME AS ABOVE												
City	Fishers	State	IN	Zip	46038	Country	US						

SEQUENCE LISTING"

<110> Witcher, Derrick
Tian, Yu
Atkinson, Paul

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Phe Trp Asn Tyr Leu Glu Arg Cys Arg Tyr Cys Asn Val Leu Cys Gly
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Ser Gln Asn Thr Gln Cys Gln Pro Cys Pro Pro Gly Thr Phe Ser Ala
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Cys Thr Ser Cys Thr Gly Phe Pro Leu Ser Thr Arg Val Pro Gly Ala
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Pro Pro Gly Thr Phe Ser Ala Ser Ser Ser Ser Ser Glu Gln Cys Gln
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Pro His Arg Asn Cys Thr Ala Leu Gly Leu Ala Leu Ile Val Pro Gly
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